

AD _____

Award Number: DAMD17-99-1-9411

TITLE: The Role of EMMPRIN in Tumor Angiogenesis and Metastasis

PRINCIPAL INVESTIGATOR: Erica Marieb
Bryan Toole, Ph.D.

CONTRACTING ORGANIZATION: Tufts University
Boston, Massachusetts 02111

REPORT DATE: May 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010716 079

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. Agency Use Only (Leave blank)	2. Report Date May 2001	3. Report Type and Period Covered (i.e., annual 1 Jun 00 - 31 May 01) Annual Summary (1 May 00 - 30 Apr 01)		
4. Title and Subtitle The Role of EMMPRIN in Tumor Angiogenesis and Metastasis		5. Award Number DAMD17-99-1-9411		
6. Author(s) Erica Marieb Bryan Toole, Ph.D.				
7. Performing Organization Name (Include Name, City, State, Zip Code and Email for Principal Investigator) Tufts University Boston, Massachusetts 02111 E-Mail: erica.marieb@yahoo.com , bryan.toole@tufts.edu		8. Performing Organization Report Number (Leave Blank)		
9. Sponsoring/Monitoring Agency Name and Address U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. Sponsoring/Monitoring Agency Report Number (Leave Blank)		
11. Supplementary Notes (i.e., report contains color photos, report contains appendix in non-print form, etc.)				
12a. Distribution/Availability Statement (check one) Approved for public release; distribution unlimited			12b. Distribution Code (Leave Blank)	
13. Abstract (<i>Maximum 200 Words</i>) (<i>abstract should contain no proprietary or confidential information</i>) A critical step in tumorigenesis is proteolytic modification of the peri-cellular matrix surrounding tumor cells by matrix metalloproteinases (MMPs). Stromal cells associated with tumors are responsible for the production of most tumor MMPs. Studies from our laboratory and those of our collaborators have shown that EMMPRIN (extracellular matrix metalloproteinase inducer), a tumor cell surface glycoprotein, stimulates the production of several MMPs by fibroblasts and endothelial cells. Antisense cDNA and ribozyme constructs were utilized in an attempt to inhibit EMMPRIN expression in TA3/ST cells, a murine breast carcinoma cell line. These constructs were not efficient in blocking EMMPRIN expression and consequently, were inactive <i>in vivo</i> . However, transfection and injection experiments done in collaboration with Dr. Stanley Zucker have shown that MDA-MB-436 human breast cancer cells transfected with GFP-EMMPRIN produce much larger tumors in nude mice than vector-transfected cells. These same EMMPRIN-transfected cells become more invasive than their control counterparts both <i>in vivo</i> and in a Boyden chamber invasion assay and they exhibit enhanced levels of MMP-2. To confirm this invasive phenotype and extend this phenomenon to non-cancerous cells, we have employed a Matrigel invasion chamber assay. MDA-MB-436, MCF-7 (mammary carcinoma) and MDCK (canine kidney epithelial) cells were either transfected with EMMPRIN or infected with a full-length EMMPRIN recombinant adenovirus and then placed in the Matrigel chambers for 24 for 48 hours. The number of cells that invaded were then counted and compared with controls. In each case, elevated EMMPRIN expression corresponded to increased invasiveness over controls. We will further explore this avenue with MCF10A and HMT-3522 S1 cells (mammary epithelial cells) as well as determine an elevation in MMPs corresponding with this phenotype.				
14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award) Breast Cancer			15. Number of Pages (count all pages including appendices) 10	
			16. Price Code	
17. Security Classification of Report Unclassified	18. Security Classification of this Page Unclassified	19. Security Classification of Abstract Unclassified	20. Limitation of Abstract Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5-8
Key Research Accomplishments.....	9
References.....	10

Introduction

A critical step in tumorigenesis is proteolytic modification of the peri-cellular matrix surrounding tumor cells by matrix metalloproteinases (MMPs). Stromal cells associated with tumors are responsible for the production of most tumor MMPs. Studies from our laboratory and those of our collaborators have shown that EMMPRIN (extracellular matrix metalloproteinase inducer), a tumor cell surface glycoprotein, stimulates the production of several MMPs by fibroblasts and endothelial cells. Antisense cDNA and ribozyme constructs were utilized in an attempt to inhibit EMMPRIN expression in TA3/ST cells, a murine breast carcinoma cell line. These constructs were not efficient in blocking EMMPRIN expression and consequently, were inactive *in vivo*. However, transfection and injection experiments done in collaboration with Dr. Stanley Zucker have shown that MDA-MB-436 human breast cancer cells transfected with GFP-EMMPRIN produce much larger tumors in nude mice than vector-transfected cells. These same EMMPRIN-transfected cells become more invasive than their control counterparts both *in vivo* and in a Boyden chamber invasion assay and they exhibit enhanced levels of MMP-2.

To confirm this invasive phenotype and extend this phenomenon to non-cancerous cells, we have employed a Matrigel invasion chamber assay. MDA-MB-436, MCF-7 (mammary carcinoma) and MDCK (canine kidney epithelial) cells were either transfected with EMMPRIN or infected with a full-length EMMPRIN recombinant adenovirus and then placed in the Matrigel chambers for 24 for 48 hours. The number of cells that invaded were then counted and compared with controls. In each case, elevated EMMPRIN expression corresponded to increased invasiveness over controls. We will further explore this avenue with MCF10A and HMT-3522 S1 cells (mammary epithelial cells) as well as determine an elevation in MMPs corresponding with this phenotype.

Body

Task 1: To show that inhibition of EMMPRIN protein expression leads to a reduction in breast tumor growth.

In an effort to show that inhibition of EMMPRIN protein expression leads to a reduction in breast tumor growth and metastasis, we constructed both human and murine EMMPRIN antisense cDNAs and ribozymes. These constructs were then transfected into TA3/ST murine breast carcinoma cells or human MDA-MB-231 human breast carcinoma cells. The stably transfected cells were then injected into the tail vein of syngeneic mice to see if growth and metastasis of the tumor would now be reduced. Neither of the constructs was efficient in blocking EMMPRIN expression, and consequently were inactive *in vivo*.

Task 2: To demonstrate a rise in MMP production by microvascular endothelial cells as well as umbilical vein endothelial cells in response to EMMPRIN treatment.

Tumor angiogenesis plays an important role in cancer progression (1). Since up-regulation of MMP activity is required for angiogenesis and tumor endothelial cells express MMPs (2,3), Dr. Stanley Zucker, in collaboration with my mentor's laboratory examined whether EMMPRIN stimulates MMP production by human endothelial cells, as well as fibroblasts. In three separate experiments, purified EMMPRIN was shown to cause a significant stimulation of stromelysin-1, gelatinase A and collagenase (MMP-1) production (but not TIMP-1 or TIMP-2) in human umbilical vein endothelial cells. The effect was most marked for stromelysin since the background levels produced by the untreated endothelial cells were low. In contrast, VEGF, an established mediator of angiogenesis, had little effect on stromelysin or gelatinase A and a relatively strong effect on MMP-1; but, the most dramatic effect of VEGF was on TIMP-1 stimulation (4). These effects were not due to influences on proliferation. Thus, EMMPRIN is a more potent inducer of MMP synthesis than VEGF.

Further evidence for a role for EMMPRIN in tumor angiogenesis has developed out of the collaboration between Dr. Zucker's and my mentor's laboratories. A line of MDA-MB-436 human breast cancer cells that grows slowly *in vivo* was transfected with EMMPRIN cDNA or with vector alone, and tested for tumor growth and metastasis in nude mice. Whereas control vector transfectants formed very small, non-metastatic tumors, the EMMPRIN transfectants in most cases formed large palpable tumors and metastasized to numerous sites. The latter tumors, but not the former, were found to be highly vascularized.

My major role in these projects has been to develop a better system for demonstrating EMMPRIN-dependent MMP stimulation in endothelial cells. I examined MMP production by

human umbilical vein endothelial cells that have been infected with a recombinant, full-length EMMPRIN adenovirus. This approach was utilized due to the availability of adenoviral reagents in the laboratory as well as ease of amplification and purification. In the past, we have used EMMPRIN purified from LX-1 cells, a human lung carcinoma cell line. The purification process is tedious, time-consuming, labor-intensive and yields small quantities of EMMPRIN. Utilizing the adenoviral approach not only provides us with another way to generate EMMPRIN, but also eliminates possible contaminants that may be in our EMMPRIN preparations purified from LX-1 cells. This approach has been shown to provide more reproducible and efficient stimulation of MMP production by fibroblasts.

In our adenoviral approach, human umbilical vein endothelial cells were infected with the EMMPRIN adenovirus. We expected that EMMPRIN would be produced by the endothelial cells and bind to its putative receptor on neighboring endothelial cells, leading to a series of signaling events that would ultimately result in MMP production. This approach failed to produce any repeatable results.

However, this methodology has led to a confirmation and extension of a homotypic interaction involving EMMPRIN on tumor cells. As previously stated, stromal cells associated with tumors are responsible for the production of most tumor MMPs. However, recent studies from our laboratory and those of our collaborators have shown that EMMPRIN not only stimulates the production of several MMPs by fibroblasts and endothelial cells, but also by the tumor cells themselves. Previous experiments in collaboration with the laboratory of Dr. Zucker have shown that MDA-MB-436 mammary carcinoma cells transfected with EMMPRIN produce much larger tumors in nude mice than vector-transfected cells. These same EMMPRIN-transfected cells become more invasive than their control counterparts both *in vivo* and in a Boyden chamber invasion assay and they exhibit enhanced levels of MMP-2 (5).

To confirm this invasive phenotype and extend this phenomenon to non-cancerous cells, we have employed a Matrigel invasion chamber assay. MDA-MB-436 (Fig. 1A), MCF-7 (mammary carcinoma) (Fig. 1B) and MDCK (canine kidney epithelial) cells (Fig. 1C) were either transfected with EMMPRIN or infected with a full-length EMMPRIN adenovirus and then placed in the Matrigel chambers for 24 or 48 hours. The number of cells that invaded were then counted and compared with controls. In each case, elevated EMMPRIN expression corresponded to increase invasiveness over controls. We will determine if this phenotype is reproducible in MCF10A and HMT-3522-S1 (mammary epithelial cells) cultures and examine the MMP levels in these cultures to see if the same MMPs that are elevated in heterotypic cultures (epithelial-stromal) are elevated in homotypic (epithelial-epithelial) cultures.

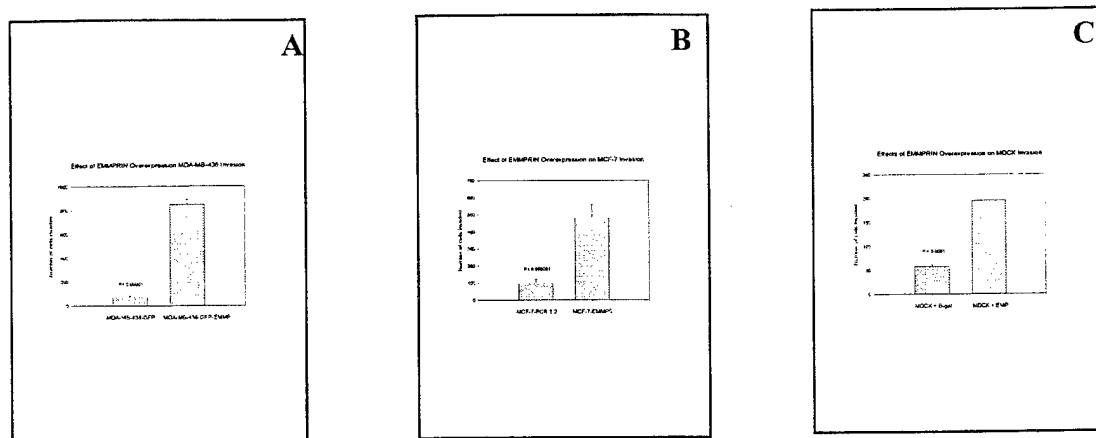


Figure 1. Effect of over-expression of EMMPRIN on cell invasion. EMMPRIN was over-expressed via transfection in MDA-MB-436 (A) and MCF-7 (B) cells and via adenoviral infection in MDCK cells (C). The cells were placed in Matrigel invasion chambers and allowed to migrate for 24 (A, C) or 48 (B) hours. Cells over-expressing EMMPRIN migrated to a greater degree than their control counterparts.

Task 3: To demonstrate endothelial cell invasion and tubule formation induced by EMMPRIN, or an augmentation of VEGF-induced endothelial cell invasion and tubule formation by EMMPRIN.

One of the systems commonly used to mimic aspects of angiogenesis involves culturing of endothelial cells on 3-dimensional gels of type I collagen or Matrigel. Under appropriate stimulus, the endothelial cells invade the gel and form capillary-like, tubular structures. In one such study (1), human umbilical vein or human dermal microvascular endothelial cells were seeded on collagen gels and treated with a phorbol ester (PMA). With both cell types, PMA treatment induced invasion of the gels and formation of vessel-like structures within the gels, along with increased levels of MMP-1 and gelatinase A. Inhibition of MMP activity especially MMP-1, prevented the induction of endothelial cell invasion and morphogenesis by PMA. These experiments imply that stimulation of MMP activity is necessary, and possibly sufficient, for induction of invasion and tubule formation in this system.

We have conducted experiments in which human umbilical vein endothelial cells were cultured in 12-well plates (5×10^5) on a type I collagen gel and treated with either bFGF, a known angiogenic agent, or purified EMMPRIN, a known MMP stimulator. Untreated cells maintain a cobblestone-like pattern, whereas both bFGF- and EMMPRIN-treated endothelial

cells form capillary-like tubules, as visualized by phase contrast microscopy. Tubule formation is such, that treating cells with 1 μ g of purified EMMPRIN yields visual results comparable to those obtained with 5ng of bFGF. We believe that the high amount of EMMPRIN needed is due to inactivation of most of the protein during purification.

Due to the constraints in purifying EMMPRIN from LX-1 cells that were mentioned above in Task 2, we utilized an adenoviral approach. The human umbilical vein endothelial cells were cultured in 12-well plates on type I collagen. Rather than adding exogenous EMMPRIN, we infected the cells with a recombinant, full-length EMMPRIN adenovirus, enabling the cells to make EMMPRIN themselves. By the same principle mentioned previously, we expected the EMMPRIN on the surface of a given endothelial cell to interact with its putative receptor on a neighboring endothelial cell, triggering a series of signaling events that will result in tubule formation, presumably by MMP stimulation. These cultures were assayed by phase contrast microscopy, Western analysis and ELISA. Infection with the recombinant, full-length EMMPRIN adenovirus failed to stimulate capillary-like tubule formation by HUVECs.

Key Research Accomplishments

- Construction of EMMPRIN antisense cDNAs and ribozymes.
- Stable transfection of TA3/ST murine breast carcinoma cells and MDA-MB-231 human breast carcinoma cells with the previously mentioned constructs.
- EMMPRIN-stimulated capillary-like tubule formation by human umbilical vein endothelial cells on type I collagen.
- EMMPRIN-dependent stimulation of MDA-MB-436, MCF-7 and MDCK invasion in Matrigel invasion chamber assays by transfection and infection with a recombinant, full-length EMMPRIN adenovirus.

References

1. Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med.* **1**: 27-31.
2. Fisher, C., Gilberston-Beadling, S., Powers, E.A., Petzold, G., Poorman, R., and Mitchell, M.A. (1994). Interstitial collagenase is required for angiogenesis in vitro. *Develop. Biol.* **162**: 499-510.
3. Polette, M. and Bierembaut, P. (1996). Matrix metalloproteinases in breast cancer. *Breast J.* **2**: 209-220.
4. Heppner, K.J., Matrisian, L.M., Jensen, R.A., and Rodgers, W.H. (1996). Expression of most matrix metalloproteinase family members in breast cancer represents tumor-induced host response. *Amer. J. Pathol.* **149**: 273-282.
5. Caudroy, S., Polette, M., Nawrocki-Rabi, B., Toole, B.P., Zucker, S. and Birembaut, P. EMMPRIN-induced invasiveness of a human mammary tumor cell line is associated with enhancement of matrix metalloproteinase-2 expression and activity. (Submitted for review)